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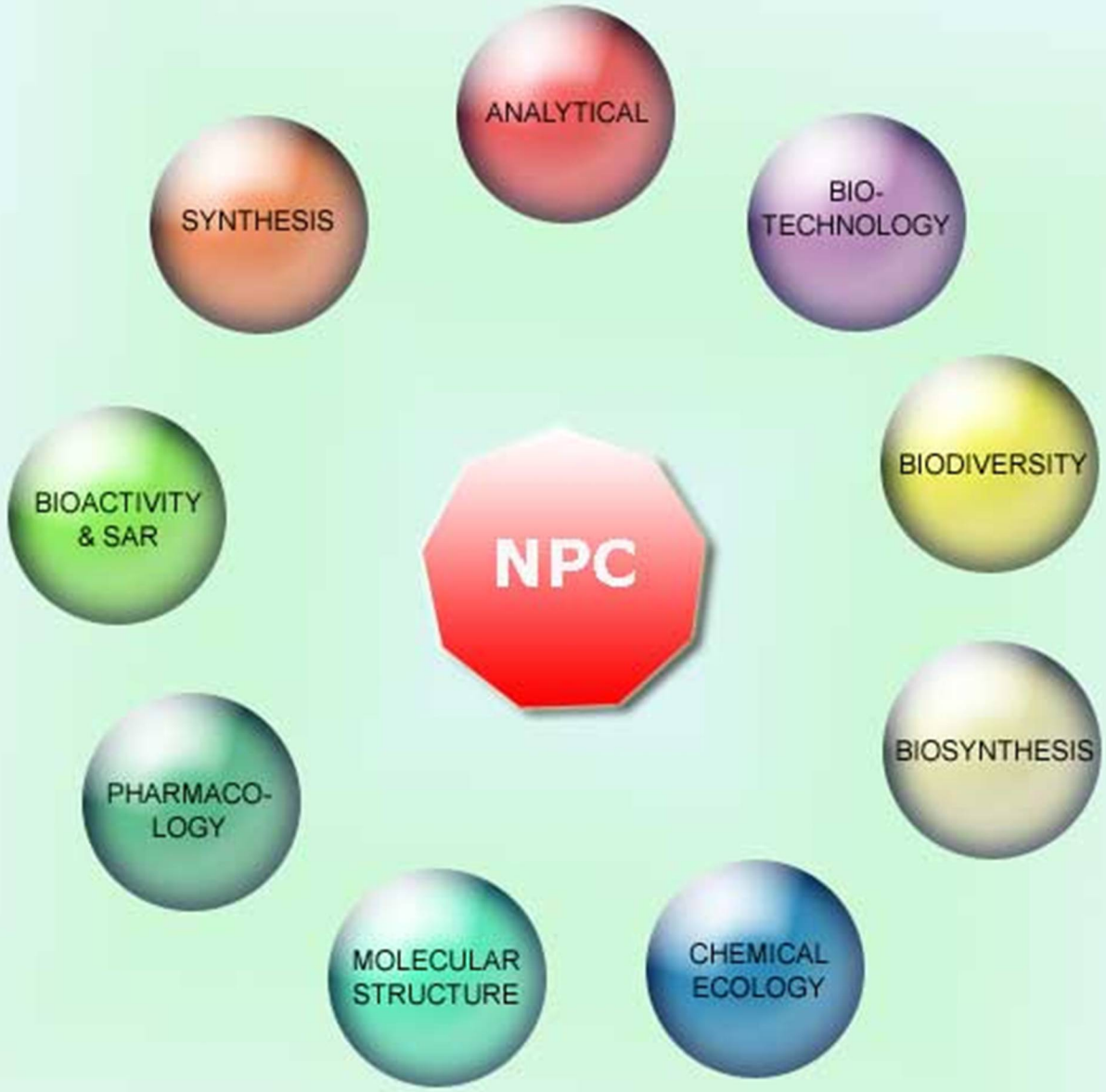

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Isolation of *C*-glycosyl Xanthones from *Coffea pseudozanguebariae* and Their Location

Pascale Talamond[1,2], Geneviève Conejero[3], Jean-Luc Verdeil[3] and Jean-Luc Poëssel[4]

[1]*UMR Diversity and Adaptability of Crops, IRD, 911, avenue d'Agropolis, BP 64501, 34394 Montpellier cedex 5, France*

[2]*Current address: UMR 226 IRD-ISEM, 361, rue Jean-François Breton, BP 5095, 34196 Montpellier cedex 5, France*

[3]*UMR BPMP 5004, UMR DAP 1098, Plate-forme d'Histocytologie et Imagerie Cellulaire Végétale, avenue Agropolis, 34398 Montpellier, cedex 5, France*

[4]*UR 1052, Genetic Improvement of Fruit and Vegetables, Domaine St Maurice, 84143 Montfavet, France*

pascale.talamond@ird.fr

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The biochemical composition of leaves from *Coffea pseudozanguebariae*, a wild caffeine-free coffee species, was determined. Two phenolic compounds were extracted from leaves, separated and characterized. Their structures were elucidated by mass spectrometry, and 1D and 2D NMR spectroscopy and were shown to be mangiferin (**1**) and isomangiferin (**2**), which were the main polyphenol products. Multiphoton fluorescence imaging was performed to visualize polyphenol distribution in leaf cross sections. Consistent biochemical analysis cell imaging techniques on leaves revealed yellow fluorescence in the epidermis and parenchyma cells corresponding to xanthone compounds.

Keywords: *Coffea pseudozanguebariae* Bridson, xanthones, fluorescence, mangiferin, isomangiferin, Rubiaceae.

Abbreviations: hydroxycinnamoyl quinic acid, HQA, caffeoylquinic acids (3,4,5), CQA; di-caffeoyl quinic acids (3,4; 3,5; 4,5), diCQA.

Coffea pseudozanguebariae Bridson, native to East Africa, is a wild species of coffee tree and the first caffeine-free species discovered in tropical East Africa. It exhibits different morphological and physiological characteristics from other species of coffee: one of the shortest fruiting times [1a], low hydroxycinnamoyl quinic acid (HQA) content [1.2% dmb (dry matter basis)] [1b], morphological aspects (small purple fruits on complete ripeness and small sized leaves), and a small sized genome (1.13 pg). Previous phytochemical investigations described the presence of alkaloids, with the major component trigonelline, diterpenes (cafestol, kahweol), and phenolic compounds (HQA, hydroxycinnamoyl quinic acid, or chlorogenic acids) in *C. pseudozanguebariae.* The unsaponifiable lipid fraction of green beans contains a mozambioside, a diterpene glycoside [2], high cafestol, kahweol, and four unknown diterpenes [3]. The diterpene glycoside, which is not present in the other species of commercial coffee trees, is the origin of the strong bitterness of this coffee [2]. In the soluble fraction, two classes of secondary metabolites have been particularly studied in green coffee beans: alkaloids and phenolic compounds. The content of the major alkaloid, caffeine, varies markedly between species, from 0%, dry wt, in *C. pseudozanguebariae* to 3.2%, dry wt, in *C. canephora* [4]. Trigonelline, a major coffee aroma compound, forms 1.02%, dry wt, of *C. pseudozanguebariae*, 0.67%, dry wt, of *C. canephora*, and 0.57%, dry wt, of *C. liberica* var. *dewevrei* [5]. Only two classes of phenolic compounds have been described in coffee plants: a major one, HQA and a minor one, proanthocyanins, from the flavonoid class [6]. Other phenolic compounds are polymeric, such as tannins. Lignans are also present in coffee seeds, although in minor amounts. This main family is formed from esters between hydroxycinnamate and quinic acid. No previous study on *C. pseudozanguebariae* has been reported in the literature. In this paper, we report the first chemical investigation of *C. pseudozanguebariae* leading to the isolation of compounds including the representative phenolic compounds and the visualization of their accumulation in the tissue.

A methanolic extract of *C. pseudozanguebariae* leaves was chromatographed on a RP-18 column and showed a major peak with a retention time at 10.1 min. This peak exhibited a spectrum with four maximum UV absorbances at 240 nm, 257 nm, 316 nm, and 365 nm. The minor peak at 10.9 min had the same UV characteristics. A peak at 7.3 min was identified, with the help of UV spectra, as 5-*O*-caffeoylquinic acid (or chlorogenic acid). Semi-preparative chromatography was used to isolate the unknown compounds. After evaporation, a yellow powder

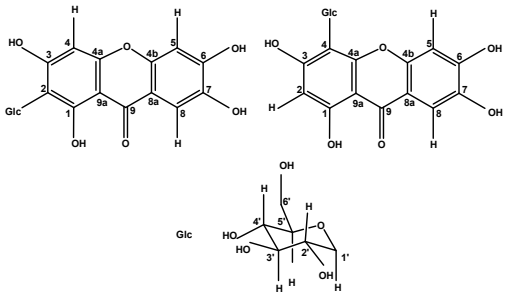

Figure 1: Structure of *C*-glucosyl xanthones. **1**: C2-β–D-glucoside 1,3,6,7 tetrahydroxyxanthen-9-one (or mangiferin), **2**: C4-β–D-glucoside 1,3,6,7 tetrahydroxyxanthen-9-one (or isomangiferin).

was obtained and used to carry out the first analysis for structure elucidation. Another separation system using a combination of cellulose column and Sephadex LH-20 gel filtration gave yellow crystalline materials (compounds **1**, **2**). From their physical, EI mass spectrometric and NMR spectroscopic features compound **1** was unambiguously established as 2-*C*-β-D-glucopyranosyl- 1,3,5,7 tetrahydroxy-xanthen-9-one (mangiferin) [7a,7b], and **2** as 4-*C*-β-D-glucopyranosyl- 1,3,5,7 tetrahydroxyxanthen-9-one (isomangiferin) [8]. Compound **2** led to a slight hypsochrome UV spectrum displacement at 318 nm. The ^{1}H spectrum of **2** is very close to that of **1** except for signals of two aromatic protons at δ_H 6.02 and 6.17.

Cross-sections of fresh *C*. *pseudozanguebariae* leaves at two stages of development were observed by epifluorescence microscopy. The young leaves (maroon color, and less than 4 cm long) showed a yellow and orange fluorescence in all cells (Figure 2A). The mature leaves (green color) did not show such a fluorescence, except for some cells in the mesophyll, which gave an orange fluorescence (Figure 2B). It is known that phenolic compounds naturally have the ability to emit fluorescence under ultraviolet light. The autofluorescence of polyphenols was used to localize them in tissues of the leaves. In order to visualize the accumulation of phenolic compounds, a spectral analysis was developed on the same fresh leaf sections. This technique was achieved with a special detection system, which enabled separation of the signals from different fluorophores. We used this technique with a multiphotonic microscope because the infra-red laser associated with this microscope gave the possibility of exciting molecules like a UV laser. With an excitation wavelength between 700 and 800 nm, it was possible to excite like a UV laser between 350 and 400 nm. In these optical conditions, spectral signatures were acquired for each pixel of the scanned image (from either leaf section or purified compound) and could subsequently be used for digital separation into component dyes. Then, from these spectral signatures, the Linear Unmix method was used to discriminate between various fluorescence signals, even with widely overlapping emission spectra. The emission spectral curve data obtained from the compounds were recorded in a spectral library and applied on cross-sections of leaves to localize them. The calculation was realized using a high number of iterations and by keeping a residual channel dedicated to pixels not corresponding to the selected spectral signatures. The spectral acquisition obtained on young leaf cross-sections showed a complex pattern of autofluorescence resulting from various fluorescent compounds. The fluorescence observed in young leaves, identified as the spectrum emission of mangiferin, was found after Linear Unmixing calculation in the upper epidermis and in some mesophyll cells (Figure 2C). The same technique was used with the spectrum emission of 5-*O*-caffeoylquinic acid (chlorogenic acid), the main phenolic compound in coffee, but no signal was detected on the leaves (data not shown). There were other uncharacterized fluorescent compounds in the mesophyll, which did not correspond to the known compounds and which seems to belong to other families of secondary metabolites. These compounds are not accumulated in the leaves of *C. arabica* and *C. canephora,* the commercial species of coffee. The decrease in yellow fluorescence observed between the very young and mature leaves may tally with biochemical analysis [9], in particular, during leaf development; there is a sharp decrease in the main phenolic compound, mangiferin. Consequently, tissue-specific localization could provide valuable information for understanding the actual role in the mechanisms of acclimatization to several environmental agents such as UV radiation or against pathogen and predator attacks. The fluorescent technologies now available allow researchers to study such dynamic processes in living cells.

Experimental

General experimental procedures: Chromatographic separation was performed using a HPLC series (Shimadzu, Prominence LC) equipped with software, a DGU-20A$_3$ degasser, an LC-20AD binary gradient pump, a SIL-20AC thermoautosampler, and a SPD-M20A diode array detector. The column used was a LiChrospher 5 µm RP18 (250X4 mm i.d.) from Merck (Darmstadt, Germany), and a guard column of the same material. The mobile phase consisted of 2mM phosphoric acid in water (eluent A) and MeOH (eluent B). The gradient program was as follows: a 25-80% MeOH gradient over 40 min at a flow rate of 0.8 mL.min^{-1}. Separation was at room temperature and the injection volume was 500 µL for isolation and 20 µL for identification. Phenolic compounds were identified by comparing their retention times with appropriate standards: 5-caffeoyl quinic acid, 3-caffeoyl quinic acid and 3,5-dicaffeoylquinic acid. A Q-TOF Micromass (Waters, Milford, MA, USA) mass spectrometer was used to obtain the MS data. The sample was solubilized in 50% water-acetonitrile solution, acidified using 0.1% trifluoroacetic acid solution and introduced at a flow rate of 5 µL min^{-1}. All the analyses were performed using an electrospray ionisation source (ESI) set to 100°C in positive ion mode with the following settings: capillary voltage -3000 V, cone voltage = 30 and 50 V, nebuliser gas (N_2) 400 L/h, desolvation temperature 120°C, drying gas (N_2) 20 l/h.

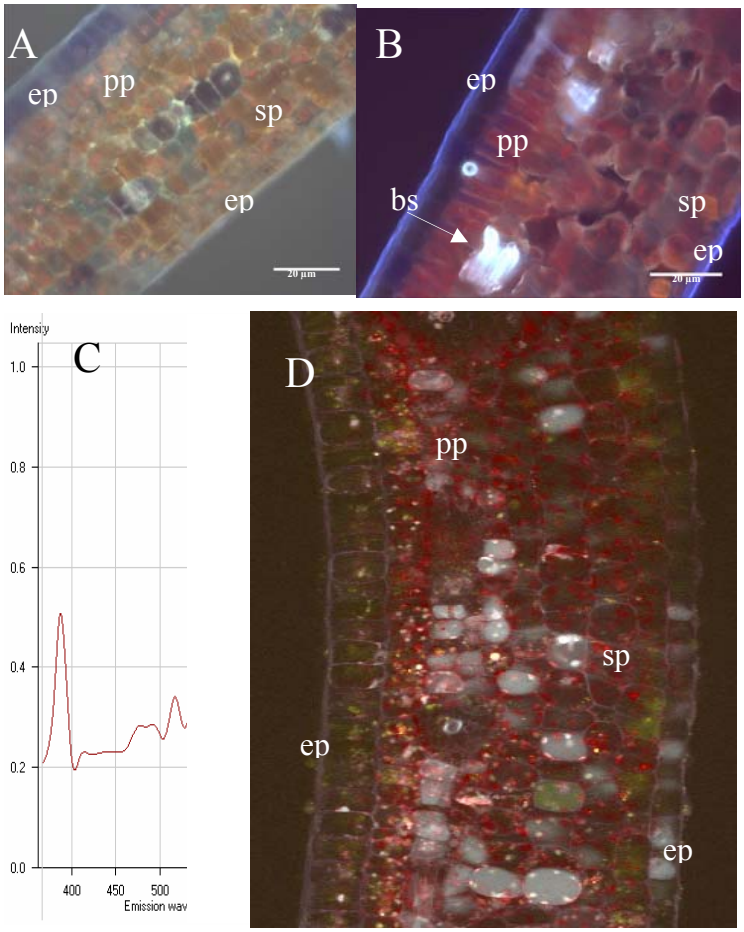

Figure 2: Histological imaging of fluorescent compound accumulation in the leaves of C. *pseudozanguebariae* . Cross-sections of young leaves (A) and mature leaves (B) by epifluorescence microscopy under UV light (long-pass filter 425 nm, real colours). Cross-sections of young leaves by spectral analysis and linear unmixing (C) on a multiphotonic microscope (false colors, yellow: mangiferin, red: chlorophyll, white: unknown). ep: epidermis, pp: palisade parenchyma, sp: spongy parenchyma, bs: bundle sheath cell, bar = 20 µm.

Mass spectra were monitored using MassLynx4.0. software, with an acquisition rate of 2 spectra s^{-1} in the range *m/z* = 70-600. NMR spectra were measured using a Bruker DRX-400 spectrometer at 400 MHz (^{1}H) and 100 MHz (^{13}C).

Histology: Thick cross-sections (50 µm) were obtained from young leaves of *Coffea pseudozanguebariae* using a vibratome (MICROM) and then dipped in 10 mM phosphate buffer saline (7 mM Na_2HPO_4, 3 mM NaH_2PO_4, 120 mM NaCl, 2.7 mM KCl). Epi-fluorescence microscopy was carried out on a Leica DMRXA equipped with a Q- Imaging camera (long-pass filter 425 nm). A Zeiss 510 META NLO multiphoton microscope equipped with a Coherent Chameleon Ultra II laser was used to obtain emission fluorescence from fresh leaves. Spectral analysis was carried out using the autofluorescence properties of polyphenol compounds without any dyes. Reference spectra on purified powder of polyphenol compounds (HQA and xanthones) were obtained from spectral acquisition with excitation at 780 nm and emission between 400 and 700 nm. The Linear Unmixing Function of the microscope (method of Emission Finger printing from Zeiss) was used to visualize the fluorescence of polyphenol compounds in cells from reference spectra.

Plant material: In these experiments we used *Coffea* plants from the collection cultivated at the IRD research centre in Montpellier (France). Leaves of *C. canephora* (DB56, DB57) and *C. pseudozanguebariae* (H65, H70) of various genotypes were taken from trees maintained in a tropical greenhouse (natural daylight, 25°C, 28°C day, 78-82% humidity). The young leaves, under 4 cm long, were cut from the tips of branches. Leaves were harvested from 5 different genotypes and 500 g of the collected leaves were frozen in liquid nitrogen immediately after collection then stored at -80°C. Each batch of leaves was freeze-dried for 48 h then stored in a cold room. *C. pseudozanguebariae* fruits were harvested at the CNRA Station in Divo (Ivory Coast) from field-grown trees.

Extraction and isolation: Fine powder of leaves and beans was obtained in an analytical grinder (IKA, yellow, A10) by grinding for 1 min, repeated 3 times. The leaf powders (2 g) were extracted with 70% MeOH solution (30 mL, X3) at 4°C and bean powders (50 mg) in 5 mL of 70%

MeOH solution. The MeOH extracts were combined, filtered and evaporated under reduced pressure to give a residue. This was suspended in MeOH and filtered through a 0.2-µm disposable filter tip-syringe assembly and directly analysed by HPLC for separation and identification. A second technique was used with 80 g of freeze-dried leaves suspended in 700 mL of MeOH-H_2O (8:2) solution at room temperature for 20 min with sonication (20 min, 24 KHz, R.E.U.S-GEX 180, Contes, France), repeating 3 times. MeOH was removed in a rotavapor. After freeze-drying, the aqueous phase was subjected to a Medium Pressure (MP) column (400X47 mm) packed with cellulose (microcristallin Avicel, Darmstadt, Germany) and successively eluted with H_2O and H_2O-MeOH (1:9). Collected fractions were purified on a Sephadex LH-20 (Fluka) column (500 X 25 mm, Fluka, Basel, Switzerland) and eluted with H_2O for compound **1**. For compound **2**, fractions were purified on a MP column (210 X 30 mm, Buchi) eluted with EtOH- H_2O (8:2). After freeze-drying, both compounds (**1** and **2**) were obtained.

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